THE ABILITY OF PGPR ISOLATES FROM IRON SAND SOIL TO PRODUCE EXOPOLYSACCHARIDES IN ATCC NO. 14 MEDIUM WITH DIFFERENT CARBON SOURCES

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Abstract. Coastal land is marginal with low soil fertility and agricultural productivity. Plant growth-promoting rhizobacteria (PGPR) that produce exopolysaccharides (EPS) can increase plant resistance and soil aggregate stability. This study aimed to determine the ability of PGPR isolates from iron sand soil to produce EPS in American Type Culture Collection (ATCC) No. 14 medium, identify the optimal carbon source, and determine the highest EPS production time for selected isolates. The effect of carbon sources on EPS production by selected isolates was studied using a Completely Randomized Design (CRD). The treatments consisted of six different carbon sources (control, glucose, fructose, sucrose, lactose, and mannitol), and each treatment was replicated three times. The data were analyzed using ANOVA and followed by the Honestly Significant Difference (HSD) test. The results showed that 16 PGPR isolates had the potential to produce EPS, with isolate PT4C5 exhibiting the highest slime index. The crude EPS production test showed that sucrose was the optimal carbon source, at 12.89 mg/L. The EPS production of isolate PT4C5 in ATCC No. 14 medium with sucrose peaked at 36 hours of incubation at 23.11 mg/L. The results also indicated that isolate type, carbon source, and fermentation time are important factors for optimizing EPS production by PGPR isolates.

Keywords: Carbon source, exopolysaccharides, iron sand soil, PGPR

1. Introduction

Marginal land has low productivity due to low soil fertility and poor aggregate stability. One example is coastal land that is vulnerable to high salinity, heavy metal accumulation, and soil structural instability, making it suboptimal for agriculture [1]. Plant Growth-Promoting Rhizobacteria (PGPR) are a solution because they can enhance plant growth and resistance to environmental stress through rhizosphere colonization [2]. PGPR can increase drought tolerance through several mechanisms, including the production of exopolysaccharides (EPS) [3-4]. The EPS produced are carbohydrate polymers located outside the cell and function as bioameliorants to improve soil aggregate stability [5,6].

EPS production by bacteria is influenced by various factors, including the availability of carbon sources [7]. The type of carbon source in the medium affects EPS production by triggering different metabolic pathways [8]. Monosaccharides, such as glucose and fructose, are readily metabolized by bacteria as energy sources. Sucrose, a disaccharide, contributes to EPS biosynthesis [9]. Lactose, as a disaccharide, is more complex and has potential for certain bacteria, such as lactic acid bacteria [10]. Mannitol, as a polyol, affects EPS production, depending on the bacteria's ability to adapt to carbon-source utilization [11]. Testing different carbon sources is necessary to determine the optimal carbon source for EPS production.

The selection of culture medium is also important in EPS production. ATCC no. 14 medium is a specific growth medium for EPS-producing bacteria, providing balanced nutrients that meet

bacterial metabolic needs [12]. This medium contains nitrogen sources and mineral salts to maintain ionic balance [13].

Microorganism growth in a liquid medium follows a growth curve pattern consisting of lag, log, stationary, and death phases. EPS production is highest during the stationary phase because bacteria experience nutritional limitations, prompting them to produce secondary metabolites, including EPS, to protect themselves from unfavorable environmental conditions [14]. EPS production reached its highest level during the stationary phase, at 36 hours of fermentation, at 10.412 g/L. The decline in EPS production after 36 hours coincided with the onset of bacterial death [15].

Several studies have shown that variations in carbon sources can affect EPS production. The best carbon source for *Pseudomonas fluorescens* PG7II.1 and *P. diminuta* PG7II.9 was sucrose at concentrations of 2 and 3% (w/v), and the weight of EPS produced was 8.04 and 1.82 mg/mL, respectively, with an incubation period of 72 hours at room temperature [13]. Sucrose at a 2% concentration produced higher EPS yields than glucose, with dry weights ranging from 5.55 to 7.55 mg/mL [7].

Iron sand is a type of marginal soil that contains metal minerals and has unique physical properties [16]. This environment enables PGPR isolates to produce EPS as an adaptive response to environmental stress. The isolates used in this study were obtained from iron sand soils on the coasts of Purworejo, Cilacap, and Kebumen. A total of 19 PGPR isolates (PT3C1, PT4C5, PT6C2, PT6P1, PT7X1, CT2P1, CT4A7, KT1Y5, KT2P3, KT3A1, KT5P1, KT6A5, KT8A6, KT8K3, KT10P2, KT11PI, PS2P1, PS2Y2, and CS3C7) exhibit strong nitrogen-fixation and phosphate-solubilization capabilities [17]. The ability of these isolates to produce EPS remains unknown, underscoring the importance of this research.

The objectives of this research were to determine the ability of PGPR isolates from iron sand soil to produce EPS in ATCC no. 14 medium, to determine the optimal carbon source for increasing EPS production by selected PGPR isolates using ATCC no. 14 medium, and to determine the highest EPS production time by selected PGPR isolates in ATCC medium no. 14 with optimal carbon sources during 72 hours of incubation.

2. Methods

2.1. Test of carbohydrate utilization as a single carbon source

A total of 1 ose was inoculated into Basal medium supplemented with each carbon source (glucose, fructose, sucrose, lactose, and mannitol) at 1% and then incubated for 24 hours at room temperature. A positive interpretation was indicated by a change in the medium from clear to cloudy (Modified from [18]).

2.2. The ability of PGPR isolates to produce EPS was tested semi-quantitatively on ATCC no. 14 agar medium

A total of one ose was inoculated by spot inoculation and then incubated for 72 hours at room temperature. EPS-producing isolates were identified by the formation of slime around the colonies. The diameter of the slime was measured using a caliper. One selected isolate with the highest slime index was used to test EPS production [19].

The index was measured using the following formula:

$$\text{Slime index} = \frac{(slime\ diameter - colony\ diameter)}{Colony\ diameter}$$

2.3. Production test of crude EPS with different carbon sources

Experimental trials on crude EPS production tests with different carbon sources were conducted using 5 mL of potential EPS-producing bacterial isolates with a density of 10^8 CFU/mL inoculated into each of 6 bottles containing 50 mL of ATCC no. 14 liquid medium. Each treatment bottle was added with one of five types of carbon sources, namely glucose,

fructose, sucrose, lactose, and mannitol, each at a concentration of 2% (w/v), and without the addition of carbon sources. The cultures were incubated in a shaker incubator at 150 rpm and 28°C for 48 hours. At the end of incubation, 500 µL of 1 mM EDTA was added to each culture to facilitate EPS release from the cell surface. The cultures were transferred to centrifuge tubes and centrifuged at 7000 rpm for 15 minutes at 4°C to separate the solid fraction from the supernatant. The supernatant obtained after separation of the bacterial cell precipitate was collected. The supernatant was then added to a cold ethanol solution at a 1:2 (v/v) ratio and incubated for 24 hours at 4°C to precipitate the EPS. After incubation, the mixture was centrifuged at 7000 rpm for 15 minutes at 4°C to separate the EPS from the supernatant. The biomass sediment in the form of crude EPS was dried in an oven at 60°C for 24 hours, and the dry EPS weight was measured. The dry EPS weight yield was calculated using the following formula:

$$\text{EPS level (mg/L)} = \frac{Dry\ EPS\ weight\ (mg)}{Media\ volume\ (L)}$$

(Modified from [7], [20]).

2.4. EPS production curve

The highest EPS production time for the selected isolate on ATCC medium no. 14, with an optimal carbon source, was achieved after 72 hours of incubation, and the culture was sampled at 0, 12, 24, 36, 48, 60, and 72 hours for growth curve construction and EPS content analysis. The growth-curve procedure and crude EPS production test using the optimal carbon source were carried out sequentially, as described previously (modified from [21]).

The survey data were analyzed descriptively. The experimental data from the EPS production test were analyzed using ANOVA at the 95% confidence level, followed by the Honestly Significant Difference (HSD) test in SPSS.

3. Results And Discussion

3.1. Utilization of carbohydrates as a single carbon source for PGPR isolates from iron sand soil

The use of carbohydrates as a carbon source is an important aspect of microbial metabolism, including that of PGPR bacteria. Carbohydrates act as the main substrate in cellular catabolism pathways to produce energy. Carbohydrates also act as precursors for the biosynthesis of important compounds, including EPS. Differences in carbohydrate chemical structures can lead to variations in the quantity and composition of EPS produced [22].

Testing the utilization of carbohydrates as a single carbon source was the first step in evaluating each isolate's metabolic activity and its EPS production potential. The results showed that all PGPR isolates from iron sand soil exhibited growth on all five carbohydrate types tested. The ability of all isolates to grow on the five carbohydrate types indicates that they possess enzymatic completeness, enabling the breakdown of both simple and complex sugars. Isolates that are able to grow well in media with certain carbon sources indicate the presence of enzymes suitable for digesting these substrates and synthesizing biomolecules. This ability reflects the presence of specific enzymatic activities, such as hydrolases or transferases, that enable isolates to degrade complex carbohydrates into simpler forms for use in metabolic processes [23]. Based on these results, the five carbon sources were used as treatments in EPS production assays to evaluate their effects on PGPR isolates.

3.2. Selection of PGPR isolates from iron sand soil in semi-quantitative EPS production

The isolates were screened early to assess their potential to produce EPS. Assessment was performed semi-quantitatively through direct observation of colonies growing on ATCC no. 14

solid medium. The focus of observation was on the morphological characteristics of the colonies, particularly the size of the slime formed (Figure 1).

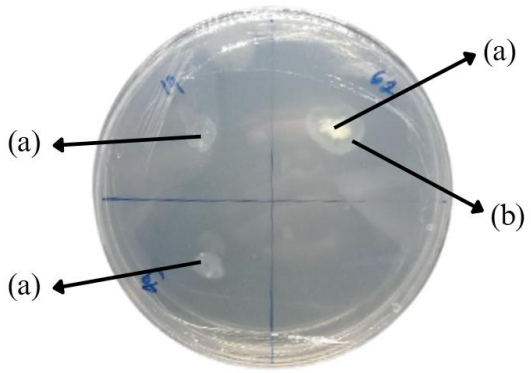

Figure 1. Morphological characteristics of EPS-producing colonies
Notes: (a) colony, (b) slime zone

Differences in slime size reflect physiological variation among isolates in their ability to synthesize and secrete EPS into the medium, resulting in slime. The slime visible on colonies is a crude form of EPS released onto the medium surface. This slime protects bacterial cells from environmental stress and enhances adhesion to surfaces via biofilm formation [24]. Colonies with a viscoelastic and wet appearance are more likely to produce higher amounts of EPS than compact and dry colonies [25].

Table 1. Colony slime index of PGPR isolates from iron sand soil on ATCC no. 14 medium

Isolate	Slime index
PT3C1	4.314 ± 1.225
PT4C5	20.022 ± 1.144
PT6C2	8.020 ± 1.660
PT6P1	11.282 ± 2.221
PT7X1	0.000 ± 0.000
CT2P1	5.531 ± 1.079
CT4A7	8.510 ± 1.905
KT1Y5	5.176 ± 1.426
KT2P3	7.619 ± 1.091
KT3A1	8.974 ± 1.175
KT5P1	10.043 ± 1.643
KT6A5	5.092 ± 1.079
KT8A6	0.000 ± 0.000
KT8K3	10.396 ± 1.736
KT10P2	6.083 ± 1.341
KT11P1	6.705 ± 1.685
PS2P1	5.458 ± 1.151
PS2Y2	0.000 ± 0.000
CS3C7	7.546 ± 1.564

Analysis of the semi-quantitative selection results showed differences in EPS production capacity among PGPR isolates from iron sand soil. The assessment was based on the slime index, which was determined from colonies on ATCC No. 14 solid medium (Table 1). This index reflects the volume of slime produced by each isolate as an initial indication of EPS biosynthesis activity. The higher the slime index value, the greater the isolate's potential to produce EPS. Colonies with a high slime index generally appear shinier, thickly slimy, and spread out, indicating EPS production. Conversely, isolates with low slime indices exhibit drier, non-slimey colonies, indicating no EPS production. These results are used as an initial visual indicator to predict EPS production potential [25]. The results showed that slime index values

varied among isolates, indicating heterogeneity in slime production ability (Table 1). A total of 16 out of 19 isolates tested produced a positive slime index on ATCC no. 14 medium. Isolate PT4C5 showed the highest slime index of 20.022 ± 1.144, indicating that isolate PT4C5 has the ability to secrete large amounts of slime onto the surface of the medium in relation to EPS production. Isolate PT4C5 was selected as the primary candidate for further EPS production testing with different carbon sources.

The results showed that 3 isolates (PT7X1, KT8A6, and PS2Y2) among 19 isolates had an index value of 0.000 ± 0.000, indicating the absence of slime around the colony. The absence of slime in these PGPR isolates may be due to a genetic inability to produce EPS or a genetic ability that is not triggered by environmental conditions. EPS expression is strain-specific and influenced by environmental conditions, including media type, pH, and nutrient availability, such as carbon sources, even though the isolates are genetically capable of producing it [26].

3.3. Production of crude EPS isolate PT4C5 from iron sand soil with different carbon sources

The production of crude EPS by isolate PT4C5 was observed based on the dry weight of EPS precipitate using ethanol from ATCC no. 14 medium without carbon sources and from medium enriched with five different carbon sources. The results showed that crude EPS production by the PT4C5 isolate varied depending on the type of carbon source added to the ATCC no. 14 medium (Table 2). Sucrose produced the highest EPS yield (0.6446 ± 0.0908 mg), followed by mannitol (0.5251 ± 0.3306 mg) and fructose (0.3117 ± 0.1863 mg). Meanwhile, glucose produced a dry EPS weight of 0.3034 ± 0.1538 mg, and lactose produced a slightly higher dry EPS weight than the control, at 0.1469 ± 0.0148 mg. The treatment without carbon sources (control) produced the lowest dry EPS weight, at 0.1387 ± 0.0356 mg. These results indicate that the PT4C5 isolate produces minimal EPS when the available carbon source is limited. In the absence of a carbon source, the EPS biosynthetic pathway is not optimally induced due to limited precursors and metabolic energy. Carbon sources are important in EPS synthesis because they directly support cell metabolism, enabling the formation of polymer chains and optimal EPS production [27].

Table 2. Average dry weight of PT4C5 isolate EPS from iron sand soil on ATCC no. 14 medium with different carbon sources

Carbon sources	Average Dry EPS Weight (mg)
Control	0.1387 ± 0.0356
Glucose	0.3034 ± 0.1538
Fructose	0.3117 ± 0.1863
Sucrose	0.6446 ± 0.0908
Lactose	0.1469 ± 0.0148
Mannitol	0.5251 ± 0.3306

The ANOVA results showed that the type of carbon source used in ATCC no. 14 medium had a significant effect on the production of crude EPS by the selected isolate PT4C5 ($p < 0.05$) (Table 3). This effect was indicated by a significance value of 0.019. These results indicate that the observed variations were not merely random fluctuations, but were actually caused by differences in treatment. The type of carbohydrate used in the medium can influence EPS biosynthesis by the PT4C5 isolate. The composition and type of carbon source in the growth medium regulate bacterial metabolism, including the extracellular polysaccharide biosynthesis pathway [28].

Table 3. Results of ANOVA testing of crude EPS production by PGPR PT4C5 isolate on ATCC no. 14 medium with different carbon sources

Sources of Diversity	Degrees of Freedom	Sum of Squares	Median Square	F Calculate	F Table (5%)	Significance
Treatment	5	248.181	49.636	4.198	3.11	0.019
Error	12	141.895	11.825			
Total	17	390.075				

ANOVA results showed significant differences, followed by an HSD test at the 95% confidence level to determine the actual differences in EPS levels among carbon sources. EPS levels (mg/L) indicate the efficiency of crude EPS production relative to the medium volume (50 mL, converted to L).

The HSD test results showed that treatment with different carbon sources significantly affected EPS production by the PT4C5 isolate. This is evident in the differences in the letter notation for the average EPS content across treatments. The highest average EPS content was observed for isolate PT4C5 with the addition of sucrose (12.8913 ± 1.8164 mg/L), which was significantly higher than the control and lactose treatments (2.7740 ± 0.7117 mg/L and 2.9387 ± 0.2959 mg/L, respectively; Table 4).

Table 4. HSD test results on the effect of different carbon sources on the average EPS content produced by PGPR PT4C5 isolate in ATCC no. 14 medium

Carbon sources	Average EPS Level (mg/L)
Control	2.7740^{a} ± 0.7117
Glucose	6.0687^{ab} ± 3.0751
Fructose	6.2333^{ab} ± 3.7268
Sucrose	12.8913^{b} ± 1.8164
Lactose	2.9387^{a} ± 0.2959
Mannitol	10.5027^{ab} ± 6.6112

Notes: Mean values followed by different letters indicate significant differences according to the HSD follow-up test at a 95% confidence level.

The treatments of glucose, fructose, mannitol, lactose, and control (Table 4) have the same or overlapping letter notation (a or ab). This notation indicates that there are no significant differences between the treatments. In contrast, sucrose has a different notation (b), indicating a significant difference from the control treatment. Therefore, sucrose is considered the optimal carbon source, significantly increasing crude EPS levels produced by the PT4C5 isolate, both statistically and numerically, with the highest value.

The type of carbon source strongly influences the metabolic efficiency of microorganisms in EPS production. The EPS level in the sucrose carbon-source treatment was the highest, at 37 mg/L. Sucrose proved to be the optimal carbon source in EPS production because its disaccharide structure allows rapid breakdown by invertase enzymes into constituent monosaccharides (glucose and fructose) that are directly used as precursors in the EPS biosynthesis pathway [11].

Sucrose enters the cell through the phosphoenolpyruvate-phosphotransferase system (PEP-PTS) pathway and is converted to sucrose-6-phosphate. This compound is hydrolyzed by the SacA/ScrB enzyme into glucose-6-phosphate and fructose. Both are converted into fructose-6-phosphate by the SacK/ScrK enzyme, which then gradually converts it into UDP-N-acetylglucosamine through a series of enzymatic reactions (GlmS, NagA, NagM, GlmU). This compound is used as a building block for EPS chains and branches via glycosyltransferase enzymes. Assembly by glycosyltransferase is followed by the addition of side sugars at the C-2/C-3/C-6 positions, resulting in branched EPS, which is then diflippased (Wzx), dipolymerized (Wzy), modified, and exported out of the cell [29].

3.4. Highest EPS production time of PGPR isolates from selected iron sand soil at optimal carbon sources

The pattern of cell growth dynamics and its correlation with EPS production were determined from the growth and EPS production curves of the selected isolate PT4C5. The medium used was ATCC No. 14, enriched with 2% sucrose as the optimal carbon source based on previous test results. The growth curve was compiled based on the calculation of the number of colonies (CFU/mL) from samples taken at each time point and displayed in the form of log CFU/mL. The EPS production curve illustrates the amount of crude EPS (mg/L) produced at the same time points. The graphs showing cell growth and EPS production are shown in Figure 2.

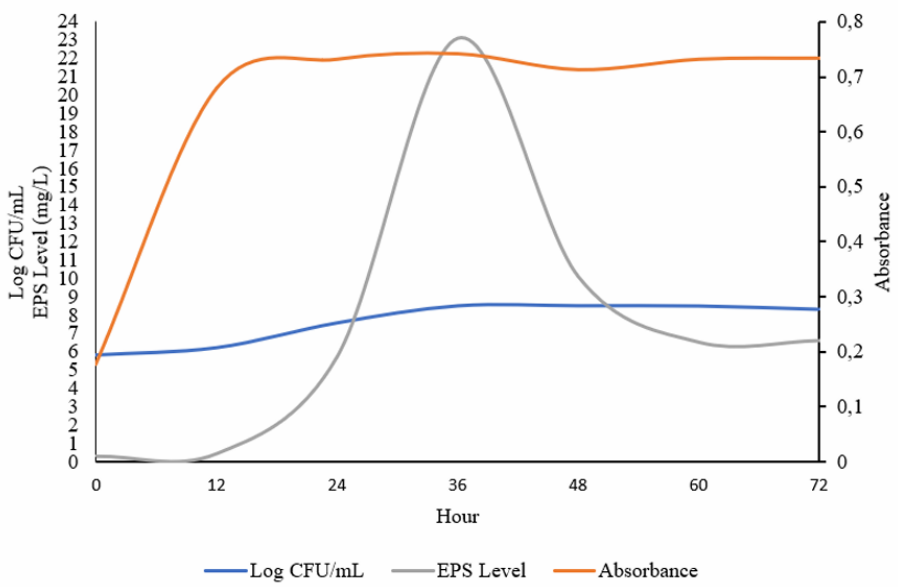

Figure 2. Relationship between growth and EPS production of PT4C5

The relationship between growth (log CFU/mL) and EPS production by isolate PT4C5 in ATCC no. 14 medium with 2% sucrose as a carbon source showed a different dynamic pattern (Figure 2). Cell growth increased gradually from the start until the 12th hour, which was the exponential phase. Bacterial growth tended to stabilize in the stationary phase between 36 and 60 hours, then decreased slightly or entered the death phase at the end of the 72-hour incubation period. The absorbance value increased rapidly up to 12 hours and remained stable thereafter, through 36 hours and to the end of the observation period. This value reflects rapid cell biomass growth initially, followed by stabilization, despite a slight decrease in the number of living cells. Meanwhile, the EPS production curve shows a sharper spike pattern. EPS levels increased significantly from the 12th hour, peaking at the 36th hour with an EPS yield of 23.11 mg/L, then decreased through the 48th hour and tended to stabilize in the final incubation phase. This indicates that EPS production does not increase linearly with log CFU/mL or absorbance, but rather correlates strongly with the late exponential to early stationary phase as a secondary metabolite [31].

During the early exponential growth phase, EPS biosynthesis is not yet active and generally begins only when bacteria enter the transition phase from stationary to death. Based on Figure 4.2, EPS production was detected at 12 hours, indicating that EPS is produced earlier than the general theory posits, which holds that EPS is a secondary metabolite produced during the stationary phase. EPS production at 12 hours is related to the utilization of sucrose as a carbon source. Sucrose is hydrolyzed to glucose and fructose, with fructose suspected to be utilized more rapidly via an efficient transport system, thereby increasing the availability of precursors for EPS biosynthesis [30].

The decline in EPS production after 36 hours is likely due to physiological and genetic factors that affect microbial metabolism. Physiological factors include EPS degradation by hydrolytic enzymes secreted during nutritional stress and EPS consumption by bacteria as an alternative carbon source when primary nutrients are depleted, because bacteria prioritize survival over growth. EPS expressed into the environment can be degraded again [32].

Factors affecting EPS production include the type of medium, substrate concentration, fermentation time, inoculum concentration, genetic traits, temperature, and pH. The medium must provide balanced nutrients to ensure optimal EPS production during the stationary phase. A Suitable inoculum concentration, temperature, and pH help maintain enzyme activity for efficient EPS synthesis [33].

4. Conclusion

Based on the research conducted, 16 of 19 PGPR isolates from iron sand soil produced EPS in ATCC No. 14 medium. Sucrose is the optimal carbon source for increasing EPS production in the PT4C5 isolate using ATCC No. 14 medium, with a value of 12.89 mg/L. The highest EPS production time for isolate PT4C5 in ATCC no. 14 medium enriched with 2% sucrose as a carbon source during a 72-hour incubation period was achieved at 36 hours, amounting to 23.11 mg/L.

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